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Recombinant Interleukin-1α, Interleukin-2 and M-CSF-1 enhance the survival of newborn C57BL/6 mice inoculated intraperitoneally with a lethal dose of herpes simplex virus-1

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Summary. Recombinant Interleukin-1α (IL-1α), Interleukin-2 (IL-2) and recombinant macrophage colony-stimulating factor-1 (M-CSF-1) as well as combinations of IL-2 and M-CSF-1 were studied for their ability to protect seven-day-old C57BL/6 mice against HSV-1 infection. Treatment of the mice with IL-2, M-CSF-1 or combinations of IL-2 and M-CSF-1 significantly increased survival rates. Treatment with IL-1α (10 U and 100 U/mouse) was most effective in protection against HSV-1, resulting in significantly increased survival rates more than four times greater than the survival rate of the infected control group.

Introduction

Disseminated herpes simplex virus-1 (HSV-1) infection of newborns is a severe condition with mortality reaching as high as 15–20% in human neonates [13]. The outcome of HSV-1 infection can be influenced by the immunocompetency of the host. Defects in the immune system of neonates, possibly resulting in increased susceptibility to HSV-1 infection, have been identified both in humans and in the murine model. In the latter, most studies have been conducted with C57BL/6 mice which, at the age of four weeks, are resistant to intraperitoneal infection with HSV-1 as adults, but as newborns are highly susceptible [15, 32, 41]. Human studies suggest a delayed production of anti-HSV-1 antibody in neonates with low antibody-dependent cellular cytotoxicity (ADCC) as compared to adults [18]. The murine model reveals that newborn mice also have poor antibody responses to exogenous antigens [22]. However, administration of large doses of anti-HSV-1 antibodies immediately before or after infection with HSV-1 may alter the result of the infection [1, 3, 8, 26, 34]. Other immunologic defects previously described in newborn mice include defective macrophage function and impaired T-cell function, characterized by altered lym-

phokine production [14, 17]. Clearance of the virus from the peritoneum following intraperitoneal (i.p.) infection is accomplished largely by peritoneal macrophages [34]. The state of activation [2] and differentiation [35] of macrophages affects their ability to restrict HSV-1 replication in vitro [31]. When mature macrophages are absent from the peritoneum, the virus is able to penetrate the central nervous system, resulting in a lethal encephalitis in the mouse model [42]. Macrophages of neonate animals are unable to restrict viral replication [17]. Numerous attempts have been made to overcome these immunological defects. The transfer of peritoneal cells from nonimmune syngeneic adult mice to newborns results in reconstitution of the ability to produce antibodies [19]. This appears to be due to both macrophages and helper T-cell populations although the latter may be replaced with soluble helper T-cell products [19]. Lethal HSV-1 infection may be prevented either by administration of macrophages and T-cells or macrophages and T-cell-lymphokine-containing fraction [14]. T-cells may also be replaced with human recombinant interleukin-2 (IL-2) [14].

IL-1 plays an important role in local and generalized inflammatory and immune responses and has a wide spectrum of biological activities, including the induction of lymphocyte proliferation and the initiation of the acute phase response [4, 6]. It also stimulates the production of IL-2, IL-3, IL-6 and the interferons [6] and acts in the augmentation of the immune response to antigens. Although originally described as a product of activated phagocytic cells, studies have shown that it is synthesized by numerous cell types. Expression of the IL-1 gene is induced in the course of antigen presentation to T-cells [21]. IL-1 activity is encoded by two different genes, IL-1α and IL-1β. The murine cDNAs were cloned and shown to detect RNA species of 2.1 and 1.4 kb, respectively, by the Northern blot technique [9, 24]. Both species share the same range of biological activities [4, 6, 36] and bind to the same 80 kDa receptor [7].

IL-2 is secreted by T lymphocytes upon stimulation with mitogen or antigen and has effects on several immune functions including enhancement of natural killer (NK) cell activity, the induction of lymphokine-activated killer (LAK) cells and stimulation of interferon-γ production [40]. It also stimulates antiviral cytotoxicity of both adult and neonate human cells [20]. Human recombinant IL-2 (rIL-2) has previously been shown to protect against acute HSV-2 genital infections in guinea pigs [40] and is an effective immune therapy in neonatal mice when administered one day prior to infection [16]. Furthermore, the production of fully differentiated T-cells has an absolute requirement for IL-2. Resting T-cells do not make IL-2 nor do they respond to external sources of the factor. Both stimulation of IL-2 production and display of the IL-2 receptor requires the introduction of antigen.

Macrophage colony-stimulating factor (M-CSF-1) belongs to a group of growth factors that stimulate proliferation and differentiation of bone marrow progenitor cells and may also stimulate mature cells. Its primary role is macrophage activation [39]. Although IL-2 protection of newborn mice from HSV-

I infection was reported [16, 17], no such studies were reported on the effect of M-CSF-1 on the resistance of newborn mice to HSV-1 infection. Since macrophages are known to possess receptors for both M-CSF-1 and IL-2 [11, 12], it is therefore possible that both cytokines might act to stimulate macrophage function. A combination of both cytokines may act synergistically to protect neonatal mice against lethal HSV-1 infection.

With the known interaction of various cytokines in the induction of the immune response in mind, we undertook the study of the protective effects of various concentrations of the recombinant cytokines IL-1α, IL-2 and M-CSF-1 and combinations of IL-2 and M-CSF-1 on the outcome of lethal HSV-1 infection in 7-day-old C57BL/6 mice.

Materials and methods

Virus

Herpes simplex virus type 1 (HSV-1) KOS strain was obtained from Prof. F. Rapp, Pennsylvania State University, Hershey, U.S.A. The virus was propagated in BSC-1 cell cultures, grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% calf serum (Beth Haemek, Israel). Mice were injected intraperitoneally (i.p.) with either 10^3 or 10^4 pfu/mouse. Under [illegible] conditions [illegible] occurred [illegible] post infection (p.i.) and tapered off by day [illegible] in most [illegible].

Mice

C57BL/6 mice, aged 7 days at the start of the experiment were obtained from the Hebrew University animal facilities in [illegible] pups. Each litter served as one experimental group. The total number of experiments with each experimental test group is indicated in Table 1.

Cytokines

IL-1α, highly purified recombinant human interleukin-1 from *E. coli* containing 5×10^4 U/ml and > 95% pure by SDS-PAGE was purchased from Genzyme Corporation (Boston, MA). IL-2 (lot LSP-805), a highly purified recombinant human interleukin-2 from *E. coli* [28, 37] containing 18×10^6 international units/ml, was 99% pure by SDS-PAGE and contained 0.012 ng/ml endotoxin by the limulus amoeba lysate assay. M-CSF-1 (lot DP-403), a highly purified recombinant human macrophage Colony-Stimulating Factor [10, 23] containing 1.24×10^8 U/ml, was > 95% pure by SDS-PAGE and contained < 0.01 ng/ml endotoxin. These cytokines were generously provided by Cetus Corporation (Emeryville, CA). Dilutions of IL-2 and M-CSF-1 were carried out in DMEM and IL-1α was diluted in sterile PBS containing 0.1% FCS as carrier protein. All cytokines were injected into the mice intraperitoneally (i.p.) at concentrations as indicated in Table 1.

Statistical analysis

Results of experiments were analyzed by χ^2 testing. Results were considered significant if $p < 0.05$.

Determination of virus in mouse brains

Brains of mice that died during the experiment were removed, homogenized in 1 ml DMEM, sonicated for 1 min and serially diluted in DMEM for titration on monolayers of BSC-1

cells in 12-well plates (Falcon). After adsorption for 1 h, monolayers were overlayed with 2% agar and 2× DMEM (1:1 vol). After incubation for 3 days at 37 °C in a humidified atmosphere enriched with 5% CO_2, monolayers were fixed in 25% formaldehyde and stained with crystal violet.

Results

Seven-day-old C57BL/6 mice were injected intraperitoneally with IL-1α, IL-2, M-CSF-1 or a combination of IL-2 and M-CSF-1 and were infected one day later with either 10^3 pfu/mouse or 10^4 pfu/mouse KOS strain HSV-1. Survival of the mice was recorded up to 21 days p.i.

Protective effect of IL-1α

The results in Table 1 (mouse group 1) show that infection of untreated one-week-old C57BL/6 mice with 10^3 pfu/mouse of the KOS strain of HSV-1 resulted

Table 1. Influence of cytokine treatment on survival rate of newborn mice infected with HSV-1 (10^3 pfu/mouse)

Mouse group	Treatment	No. of experiments[a]	Survival[b]	%	p-value[c]
1	Control: HSV-1 KOS	3	6/30	20	
2	10 U IL-1α[d]	3	18/20	90	< 0.0001
3	100 U IL-1α[d]	3	29/30	97	< 0.0001
4	1000 U IL-1α[d]	1	3/10	30	0.51
5	Control: HSV-1 KOS	5	16/48	33	
6	6×10^3 U IL-2[e]	4	23/38	61	0.0119
7	6×10^4 U IL-2[e]	5	27/50	54	0.0393
8	10^3 U M-CSF-1[f]	4	21/39	54	0.0543
9	10^4 U M-CSF-1[f]	4	9/38	24	0.33
10	10^5 U M-CSF-1[f]	3	12/25	48	0.22
11	10^6 U M-CSF-1[f]	2	6/16	38	0.76
12	10^6 U M-CSF-1 × 2[g]	2	6/19	32	0.89
13	6×10^3 U IL-2 + 10^3 U M-CSF-1[h]	2	15/20	75	0.0017
14	6×10^4 U IL-2 + 10^6 U M-CSF-1[h]	4	30/39	77	0.0001

[a] In each experiment one litter (10 newborn mice) was treated with the cytokines and subsequently inoculated with HSV-1 (KOS) at 10^3 pfu/mouse

[b] Total number of survivors on day 21 p.i. per total number of experimental animals in each group

[c] χ^2 tests (groups 2 to 4 relative to control group 1, groups 6 to 14 relative to control group 5)

[d] Animals received single doses of IL-1α as indicated 24 h prior to virus infection

[e] Animals received single doses of IL-2 as indicated 24 h prior to virus infection

[f] Animals received single doses of M-CSF-1 as indicated 24 h prior to virus infection

[g] Animals received single doses of M-CSF-1 as indicated

[h] Animals received single doses of IL-2 and M-CSF-1 as indicated 24 h prior to virus infection

in a survival rate of 20% at 21 days p.i. Treatment of the neonates with IL-1α at doses of 10 U/mouse or 100 U/mouse resulted in a highly significant increase in survival rates to 90% (mouse group 2, $p < 0.0001$) and 97% (mouse group 3, $p < 0.0001$), respectively. IL-1α at doses of 1000 U/mouse resulted in a survival rate of 30% (mouse group 4, $p = 0.51$). These results are presented graphically in Fig. 1 A. Statistical comparison of the survival rate obtained after treatment with 10 U/mouse (mouse group 2) versus 100 U/mouse (mouse group 3) IL-1α shows $p = 0.33$, an insignificant difference (Table 2). However, comparison of mouse group 2 (10 U/mouse IL-1α) with group 4 (1000 U/mouse IL-1α) or group 3 (100 U/mouse IL-1α) with group 4 reveals highly significant p values of $p = 0.0007$ and $p < 0.0001$, respectively.

Protective effect of IL-2

The results for mouse group 5 (another set of controls) show that infection of untreated one-week-old C57BL/6 mice with 10^3 pfu/mouse of the KOS strain of HSV-1 resulted in a survival rate of 33% at 21 days p.i. Treatment of the neonates with either 6×10^3 U/mouse or 6×10^4 U/mouse of recombinant IL-2 (rIL-2) one day prior to infection (mouse groups 6 and 7, repeated 4 and 5 times respectively) resulted in a statistically significant increase in survival with survival rates of 61% ($p = 0.0119$) and 5% ($p = 0.0395$) respectively (represented in Fig. 1 B). The difference in survival rates between mouse groups 6 and 7 was not significant ($p = 0.64$).

Protective effect of M-CSF-1

Treatment with 10^3 U/mouse recombinant human M-CSF-1 (group 8, repeated four times) also resulted in a significant increase in survival to 54% ($p = 0.0543$), as compared to the survival of the untreated infected controls (group 5) (Fig. 1 C). Treatment with M-CSF-1 at doses of 10^4 U/mouse or 10^6 U/mouse (mouse groups 9, 10 and 11) or with two individual doses of M-CSF-1 10^6 U/mouse one day prior to and one day after infection (mouse group 12) showed no significant difference in survival rates relative to the control. In fact, administration of 10^4 U/mouse M-CSF-1 had an apparently deleterious effect, decreasing survival from 33% to 24% (mouse group 9). The reason for this effect is not known.

Protective effect of combinations of IL-2 and M-CSF-1

Injection of both IL-2 and M-CSF-1, administered one day prior to infection (mouse groups 13 and 14) significantly increased survival rates more than two-fold (to 75% survival, $p = 0.0017$), with no significant difference between a combination of 6×10^3 U/mouse IL-2 and 10^3 U/mouse M-CSF-1 (mouse group 13) or one of 6×10^4 U/mouse IL-2 and 10^6 U/mouse M-CSF-1 (mouse group 14) ($p = 0.87$) (results are represented graphically in Fig. 1 D). While a statistical comparison of the increase in survival rates between groups receiving 6×10^3

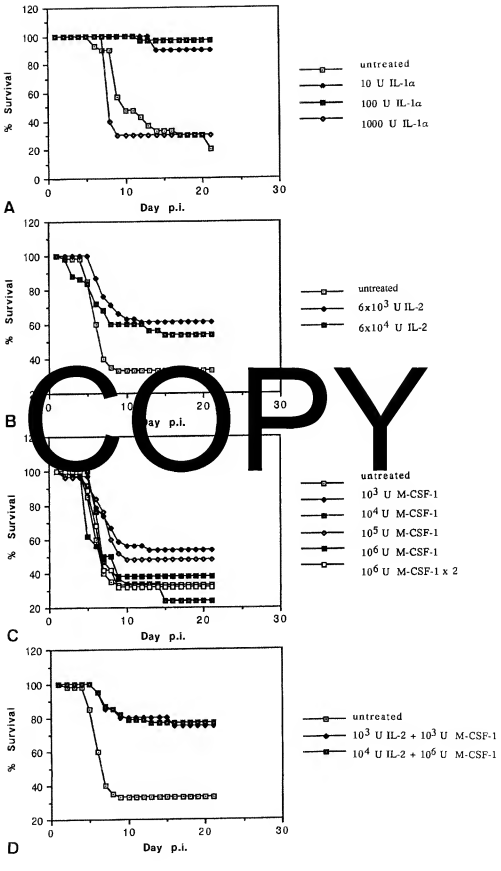
% Survival
Day p.i.
untreated
10 U IL-1α
100 U IL-1α
1000 U IL-1α
A
% Survival
Day p.i.
untreated
6x10³ U IL-2
6x10⁴ U IL-2
B
% Survival
Day p.i.
untreated
10³ U M-CSF-1
10⁴ U M-CSF-1
10⁵ U M-CSF-1
10⁶ U M-CSF-1
10⁶ U M-CSF-1 x 2
C
% Survival
Day p.i.
untreated
10³ U IL-2 + 10³ U M-CSF-1
10⁴ U IL-2 + 10⁶ U M-CSF-1
D

Table 2. Statistical comparison of survival rates of newborn mice pretreated with cytokines and infected with HSV-1

Mouse group[a]	Treatment	Survival		p-value[b]
		surv./total	%	
2	10 U IL-1α	18/20	90	0.33
3	100 U IL-1α	29/30	97	
2	10 U IL-1α	18/20	90	0.0007
4	1000 U IL-1α	3/10	30	
3	100 U IL-1α	29/30	97	< 0.0001
4	1000 U IL-1α	3/10	30	
6	6×10^3 U IL-2	23/38	61	0.27
13	6×10^3 U IL-2 + 10^3 U M-CSF-1	15/20	75	
7	6×10^4 U IL-2	25/50	54	0.025
14	6×10^4 U IL-2 + 10^6 U M-CSF-1	30/39	77	

[a] Mouse groups same as in Table 1
[b] χ^2 tests

IL-2 alone or a combination of 6×10^3 IL-2 and 10^3 U M-CSF-1 reveals an insignificant difference ($p = 0.33$, Table 2). A comparison between the groups which received 6×10^4 U IL-2 and those which received a combination of 6×10^4 U IL-2 and 10^6 U M-CSF-1 shows $p = 0.025$, a moderately significant difference.

The effect of cytokines on the outcome of infection of one-week-old C57BL/6 mice with 10^4 pfu/mouse of the KOS strain of HSV-1 was also studied. This virus dose killed all the infected, untreated newborn mice. Treatment of the newborn mice with rIL-2 at 6×10^4 U/mouse prior to infection resulted in a highly significant increase in survival to 70% of the mice ($p = 0.0000$, to four decimal places) while M-CSF-1 at 10^3 U/mouse protected 30% of the mice ($p = 0.008$). A combination of rIL-2 at 6×10^4 U/mouse and M-CSF-1 at 10^6 U/mouse protected 60% ($p = 0.001$) of the mice. These results show that cytokine treatment is of protective value against a highly lethal dose of HSV-1.

Fig. 1 A–D. Effect of cytokine treatment on the survival of newborn C57BL/6 mice infected with HSV-1. Mice were injected intraperitoneally with various doses of cytokines 24 h before intraperitoneal infection with 10^3 pfu/mouse of HSV-1 (KOS strain). Results are expressed in terms of percent survival. A Protective effects of IL-1α treatment. B Protective effects of IL-2 treatment. C Protective effects of M-CSF-1 treatment. D Protective effects of combined cytokine treatment

Fate of virus in cytokine-treated mice

Virus content in brains from those newborn mice that were infected with HSV-1 and died during the observation period of 21 days was determined. Virus plaques were isolated from brain homogenates of the majority of infected newborn mice that succumbed to the virus infection, indicating that viral encephalitis was indeed the cause of death. Virus titres as high as $10^3 - 10^4$ pfu/ml of brain suspension were recorded. This result is consistent with the observation of hind leg paralysis in many mice prior to death, indicating that the infecting virus penetrated the adrenal gland and the spinal cord and also infected the central nervous system [27]. No infectious virus was found in the brains of the HSV-1-inoculated neonatal mice that were protected by cytokines and survived the virus infection.

Discussion

The increased susceptibility of neonate animals and humans to lethal infection with HSV-1 may be due to a series of immunologic defects including defective macrophage function (with macrophages unable to process antigens) and impaired T-cell function. The role of T-cells may be explained, at least in part, with IL-2

Treatment with rIL-2 24 h prior to infection with HSV provided the highest degree of protection, increasing survival to more than four times that of the control group. This 24 h period needed to confer resistance of the newborn mice to HSV-1, coincides with the time period required for the protection of mice against *Pseudomonas aeruginosa* infection [37]. This time period allows IL-1 to activate T_h lymphocytes and macrophages, leading to an effective control of HSV-1 virus infection and spread in over 90% of the infected newborn mice.

Previous studies by Kohl et al. [16] have demonstrated that administration of recombinant human IL-2, optimally at doses of 100 U given one day prior to infection [17], provides effective immune therapy for neonatal HSV infection. Our results differ from those of Kohl [16, 17] regarding optimal dose of IL-2. We found rIL-2 at doses of 6×10^4 U/mouse to give maximal protection to neonate mice when administered one day prior to infection with HSV-1. However, differences in protection afforded by IL-2 at 6×10^3 U/mouse versus IL-2 at 6×10^4 U/mouse were not significant. An adjustment in the survival rate of mice treated with IL-2 at 6×10^4 U/mouse should be made as one set of ten mice died by day 6 post-infection and no virus was isolated from brain homogenates. However, a lethal infection of the adrenal glands cannot be ruled out as the cause of death prior to entry of the virus to the brain. If this group of ten mice is excluded from the calculations, we obtain a survival rate of 68%, instead of 54% (mouse group 7, Table 1).

Our results with M-CSF-1 revealed a slight increase in the survival rate of the newborn mice treated with the cytokine at doses of 10^3 U/mouse, but not at higher doses. Our findings suggest that M-CSF-1 stimulation of peritoneal

macrophages enhances the survival of the newborn mice and protect half of each litter against a lethal infection with HSV-1. Thus, stimulation of the immature peritoneal macrophages by M-CSF-1 has a protective value similar to that of IL-2 stimulation of immature T-cells in the newborn mice.

In this study, we showed that combinations of the recombinant cytokines IL-2 and M-CSF-1 that stimulate the immature T-cells and macrophages, respectively, provide a high degree of protection against infection with HSV-1. Survival rates greater than two times that of the control group were observed, suggesting that these two cytokines act synergistically to increase resistance of the newborn mice to HSV-1 infection. Yet, despite the marked ability of a combination of these cytokines to protect against infection, survival rates reached a maximum of only 77%, indicating that even these combination doses are not sufficient to confer full protection. Increasing the concentration of either cytokine in the combination dose appears to be ineffective, or even deleterious, (based on our results), thus the use of an additional cytokine such as IL-1α might be used to further increase survival rates. The successful protection of newborn C57BL/6 mice against HSV-1 by recombinant IL-1, as shown in the present study, might be taken to suggest that induction of the maturation of macrophages and helper cells requires the induction of intracellular molecular events in these cells which lead to the maturation of the cells and to their antiviral activity.

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